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Proteins of the lactococcin A secretion system: *lcnD* encodes two *in-frame* proteins

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Abstract

Polyclonal antibodies were raised against LcnC and LcnD proteins of the *Lactococcus lactis* bacteriocin lactococcin A secretory system to examine their cellular location and interaction. Two major reacting bands were detected by Western immunoblot with the anti-LcnD antibody: one of 52 kDa (LcnD) and another of 45 kDa, called here LcnD*. LcnD* was still detectable after removing the AUG start codon for LcnD. Chemical cross-linking analyses of membrane fractions of *L. lactis* cells expressing the LcnC/D secretion machinery were performed. Our results indicate that LcnD is present in the secretion machinery complex as a dimer and is able to interact with LcnD* and LcnC.

Keywords: Lactic acid bacterium; Bacteriocin; Lactococcin A; Protein secretion; *Lactococcus lactis*

1. Introduction

Lactococcin A (LcnA) is a narrow specificity bacteriocin produced by certain strains of *Lactococcus lactis* [1,2], a member of the lactic acid bacteria (LAB) family. These bacteria are of eminent economic importance because of their widespread use in food and feed fermentations and their potential to produce a number of substances with antimicrobial activity which can be used as biopreservatives.

Secretion and maturation of LcnA has been shown to depend on the two membrane proteins LcnC and LcnD that, together, form a Type I (*sec*-independent) secretion system [3]. LcnC belongs to the family of ABC transporters, and LcnD is proposed to be the accessory protein [1]. The structural genes *lcnC* and *lcnD* were identified in an operon upstream of the bacteriocin operon on a plasmid [1], but homologues of *lcnC* and *lcnD* were also shown to be chromosomally located in *L. lactis* IL1403, a strain that does not produce bacteriocin activity [4].

The membrane topology of LcnD conforms to that of accessory proteins of Type I transport systems: they are bitopic proteins with a short N-terminal part residing in the cytoplasm, one trans-membrane domain and the C-terminal part, representing the majority of the protein, at the extracellular side of the cytoplasmic membrane [5]. The function of the accessory proteins of ABC transporters in Gram-positive bacteria is unknown.

To be able to exploit the full potential of bacteriocins in food preservation, fundamental knowledge is needed not only of their mode of action and inhibitory spectra, but also of the secretory machinery. In this study, we describe a new factor involved in the secretory process and examine the protein interactions involved in LcnA secretion by protein cross-linking studies.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *L. lactis* was grown as standing cultures at 30°C in 2-fold diluted M17 broth [6], supplemented

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Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties/genotype[a]	Source or reference
L. lactis subsp. *cremoris* MG1363	Plasmid-free	[19]
L. lactis subsp. *lactis* IL 1403	Plasmid-free, chromosomally encoded secretion/maturation machinery for LcnA	[20]
E. coli C41(DE3)	BL21(DE3) derivative	[21]
Plasmids		
pKV4	Em^R, pIL253 derivative carrying *lcnC*, *lcnD*, *lcnA*, *lciA*, *lcnB*, *lciB*	lab collection
pMG36A	Cm^R, pMG36 derivative carrying *lcnA* and *lciA* under P32	lab collection
p*lcnD*	pMG36A carrying *lcnD* under control of P32	this study
p*lcnDΔ*	pMG36A carrying *lcnD* mutated in the AUG codon	this study
pL46	pMG36A carrying *lcnD* mutated in the 46th codon	this study
pET32	Histidine fusion vector	lab collection
pET32*lcnD*	pET32 derivative carrying *lcnD*	this study
pET32$lcnC_{200}$	pET32 derivative carrying $lcnC_{200}$	this study

[a]Cm^R: chloramphenicol resistance; Em^R: erythromycin resistance; P32: lactococcal constitutive promoter.

with 0.5% glucose (G1/2 M17). For *L. lactis*, chloramphenicol and erythromicin were used at final concentrations of 4 and 5 μg ml^{-1}, respectively. *Escherichia coli* was grown in TY medium [7] at 37°C with aeration. Chloramphenicol and ampicillin were used at final concentrations of 10 and 100 μg ml^{-1}, respectively. Isopropylthio-β-D-galactoside (IPTG) was used at a final concentration of 1 mM.

2.2. Molecular cloning and plasmid construction

General molecular cloning techniques were performed essentially as described by Sambrook et al.[7]. DNA was isolated as described before [8,9]. Electrotransformation of *L. lactis* was performed as described earlier [10].

Deletion of the *lcnD* start codon was performed by PCR with oligonucleotides *lcnDΔ*-5′-AAGCTTAAGCTTTTT-GATAAAAATTACTG-3′; and *lcnD*(Asp718) 5′-GG-TACCGGTA CCCTCTACTGATTGCCTCTTCCC-3′, the resulting fragment was cloned in pMG36A. Site-directed mutagenesis of *lcnD* was performed by double-step PCR with the oligonucleotides *lcnD*(RBS) 5′-CCGGAATGCATGGGTCGACGCAC AAAATGGC-3′ and *lcnD*46TTA 5′-GTACTTATTACGG**TTA**ACTCCT-TATGGGCAAA G-3′ (the mutated codon is indicated in bold). Plasmids pET32$lcnC_{200}$ and pET32*lcnD* were constructed by cloning in the *Nco*I–*Eco*RI sites of pET32 respectively the first 600 bp of *lcnC* obtained by PCR amplification using as primers *lcnC*Nco-AA**C-CATGG**GTA AATTTAAAAAGAAA and $lcnC_{200eco}$-AA-**GAATTC**GGCATTTGGAATATAG, and *lcnD* gene obtained by PCR amplification with primers *lcnD*Nco-AA**CCATGG**GGCATGGGT CGACGC and *lcnD*Eco-AA**GAATTC**GGTACCC TCTACTGATTGCCTCTTC-CC.

Plasmid p*lcnD* was constructed by cloning in the *Hin*dIII–Asp718 restriction sites of pMG36A the *lcnD* gene obtained by PCR amplification using the primers *lcnD*H-5′-CC**AAGCTT**GGAATGCATGGGTCGACGCACAA-AATGGC-3′ and *lcnD*(Asp718) 5′-CC**GGTACC**CTC-TACTGATTGCCTCTTCCC-3′.

2.3. Overexpression and partial purification of hexa-His-tagged LcnC/D proteins

E. coli C41(DE3) cells [11], carrying plasmid pET32$lcnC_{200}$ or pET32*lcnD* were treated with 1 mM IPTG. Cells were harvested by centrifugation (10 000 × *g*, 4°C, 10 min) and passed twice through a French pressure cell (18 000 p.s.i., on ice). The supernatant was adjusted to 60 mM imidazole final concentration, pH 8.0, and applied onto a nickel chelating column. His-tagged $LcnC_{200}$ or LcnD proteins were eluted with a buffer containing 50 mM NaPi buffer and 500 mM imidazole (pH 6.0) and used to raise rabbit polyclonal antibodies.

2.4. Cell fractionation and SDS–PAGE

To fractionate *L. lactis* cells, protoplasts were disrupted using a French pressure cell (two cycles at 18 000 p.s.i., on ice). Subsequently, samples were centrifuged for 10 min at 4°C (1000 × *g*) to remove intact cells, debris and inclusion bodies. The supernatant was collected and centrifuged at 135 000 × *g* for 30 min at 4°C and the resulting supernatant was designated the cytoplasmic fraction. The pellet was the membrane fraction.

Protein samples for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) were prepared and loaded on gel as described earlier [12]. Proteins were blotted onto PVDF membranes. Polyclonal antibodies against LcnC and LcnD were used as primary antibodies; mouse horseradish peroxidase-conjugated antirabbit immunoglobulins were used as secondary antibodies. In vivo formaldehyde cross-linking was performed as previously described [13].

2.5. LcnA activity test

LcnA activity was determined by the dilution assay as described earlier [14], except that microtitre plates with wells containing 200 μl of $G_{1/2}$M17 broth were used. *L. lactis* IL1403 was used as indicator strain: in each well of the microtitre plate, 100 μl of a 10^4-diluted overnight cul-

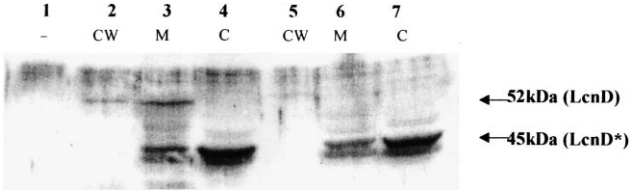

Fig. 1. Western hybridisation of cell fractions of *L. lactis* MG1363(p*lcnD*) (lanes 2–4) and MG1363(p*lcnD*Δ) (lanes 5–7), separated by SDS–(10%)PAGE. Cell wall (CW) fractions were loaded in lanes 2 and 5, the membrane (M) fractions in lanes 3 and 6, while lanes 4 and 7 contain cytoplasmic (C) fractions. Lane 1: cell-free extract of MG1363(pMG36A), used as negative control. The equivalent of 10μl of cells (late exponential phase) were applied in each well. Sizes of LcnD and LcnD*, as determined from a comparison with the protein molecular mass marker on the same gel (not shown), are indicated in the right margin. Polyclonal antibodies raised against LcnD were used as primary antibodies.

ture of this strain containing approximately 10^4 colony forming units was applied.

3. Results and discussion

3.1. Anti-LcnD antibodies react with two proteins

Anti-LcnD polyclonal antibodies were used to examine cell-free extracts of *L. lactis* MG1363(p*lcnD*). After Western immunoblot analysis, two major reacting bands were detected, one of 52 kDa that corresponds to the previously identified LcnD [1] and another one of 45 kDa (Fig. 1, lanes 3 and 4). As both bands are absent in cell-free extracts of MG1363(pMG36A) (Fig. 1, lane 1), the band at 45 kDa is not the result of unspecific antibody reaction. In fact it is a product of the *lcnD* gene and was labelled LcnD*.

To understand the nature and the cellular location of the previously unknown smaller protein, cell fractionation studies on MG1363(p*lcnD*) cells were performed. LcnD was only present in the membrane fraction (Fig. 1, lane 3), while LcnD* was localised mainly in the soluble cytoplasmic fraction (Fig. 1, lane 4).

Because LcnD is a monotopic membrane protein with the membrane-spanning domain formed by the amino acid residues 22–40 [5], it is likely that LcnD* lacks at least the first 40 residues of LcnD. LcnD* could have been formed either by specific degradation of LcnD or by alternative translation initiation of *lcnD* mRNA. In the latter case the alternative start codon should be located after the 40th codon.

3.2. LcnD* is not a degradation product of LcnD

To understand the origin of the LcnD* protein, the AUG start codon of *lcnD* was removed (Section 2). As expected, *L. lactis* carrying the mutated *lcnD* gene on a plasmid, strain MG1363(p*lncD*Δ), did not produce the 52-kDa protein LcnD (Fig. 1, lane 6). The strain did produce LcnD*, however, and this truncated protein was mostly present in the cytoplasmic fraction (Fig. 1, lane 7). This result indicates that LcnD* is not a proteolytic product of the larger protein, LcnD. Apparently LcnD* is produced from the *lcnD* mRNA by translation from an internal start codon.

We identified the UUG triplet of leucine 46 as a possible alternative translational start codon. This codon is immediately downstream of a putative Shine Dalgarno (SD) consensus sequence (Fig. 2). Direct evidence that the Leu46 UUG codon is the start codon of *lcnD** was obtained by site-directed mutagenesis. Using PCR the UUG codon was changed into UUA (Section 2), an alternative codon for Leu but not a start codon.

Again, MG1363(p*lcnD*) produced LcnD and LcnD* (Fig. 3). The protein extract from the MG1363 strain carrying plasmid pL46 with the *lcnD* gene in which the UUG codon has been mutated contained the LcnD band, but not that corresponding to LcnD* (Fig. 3). Apparently LcnD* translation starts form the UUG codon in position

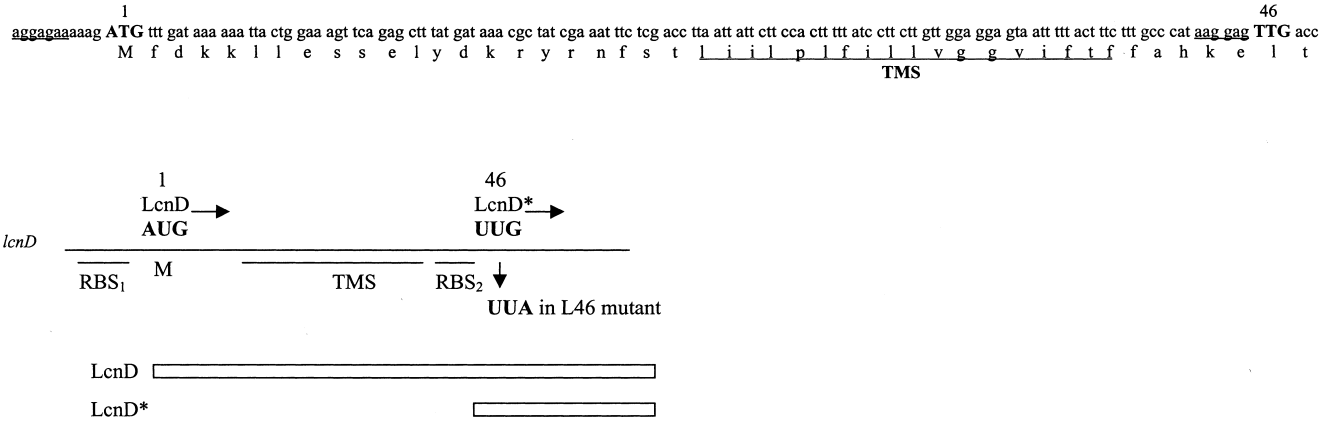

Fig. 2. Schematic presentation of the nucleotide and derived amino acid sequences of LcnD. The potential RBSs are underlined, the putative start codons are in bold and in upper case. The underlined amino acid residues (from 22 to 40) represent the trans-membrane segment (TMS) of LcnD (9) which is absent in LcnD*. Below is indicated the correspondence between *lcnD* mRNA and the two *in-frame* proteins.

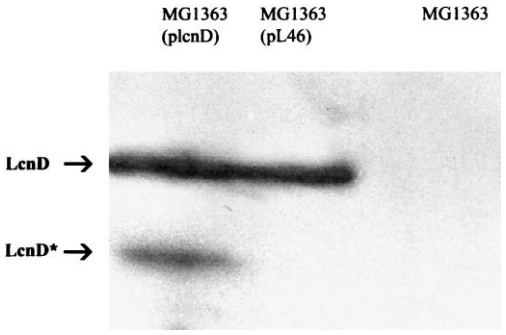

Fig. 3. Western hybridisation of cell-free extracts of MG1363(p*lcnD*), MG1363(pL46) and MG1363 used as negative control. 15 μg of protein were loaded in each lane and run on a SDS 10% PAA gel. Sizes of LcnD and LcnD*, as determined from a comparison with the protein molecular mass marker on the same gel (not shown), are indicated in the left margin. Polyclonal antibodies against LcnD were used as primary antibodies.

46, which explains the cytoplasmic location of LcnD*, as the truncated protein lacks the N-terminal TMS of LcnD.

The gene of the accessory protein for colicin V secretion in *E. coli*, *cvaA*, also encodes two *in-frame* products, namely CvaA and CvaA* [15]. CvaA is an integral membrane protein [16]. CvaA* lacks the hydrophobic residues present in the N-terminal region of CvaA and is localised in the cytoplasm [15]. Although CvaA* is not essential for ColV secretion, it stabilises CvaA, thereby enhancing ColV secretion [15]. A model has been proposed in which CvaA* interacts with the secretion machinery from the cytoplasmic side of the membrane [15].

3.3. Effect of lcnD mutations on LcnA production

L. lactis IL1403 has a chromosomal copy of the *lcnC/D* operon. The products LcnC′ and LcnD′ are able to secrete LcnA [4]. Il1403 was used to analyse the effect of the presence of LcnD* on LcnA production (Table 2).

IL1403 itself does not produce any bacteriocin; plasmid pKV4, carrying the *lcnC/D* genes and the structural (*lcnA*) and immunity (*lciA*) genes for LcnA, was introduced in IL1403 by electroporation. IL1403(pKV4) produces approximately 5120 arbitrary units (AU) of LcnA. IL1403(pMG36A), in which only the *lcnA* and *lciA* genes are located on the plasmid, produces approximately 1280 AU. This amount of bacteriocin is, thus, secreted by the chromosomally encoded secretion machinery (LcnC′ and LcnD′). IL1403(p*lcnD*), a strain which carries, apart from the chromosomal *lcnD′*, extra copies of the *lcnD* gene on the plasmid, produces the same amount of LcnA as IL1403(pMG36A). This result indicates that the chromosomally encoded LcnC′ should be the limiting factor for LcnA secretion. IL1403(pL46), in which the plasmid copy of *lcnD* specifies LcnD but not LcnD*, produces as much LcnA as that specified by pMG36A and p*lcnD*. Interestingly, the strain IL1403(p*lcnD*Δ), in which LcnD* is encoded from the plasmid-located *lcnD*, but which does not form LcnD, produces far less bacteriocin than the other strains tested (160 AU).

The differences in the amounts of LcnA produced by the various strains we used, indicate that LcnD* is not able to interact with the chromosomally encoded LcnD′ protein with the same affinity as it has for LcnD; indeed LcnD* has a negative effect on LcnA production in the strain IL1403(*plcnDΔ*). The latter result is in favour of a chaperone function for LcnD*: the bacteriocin would be titrated away by LcnD* and is not available for the LcnC′/D′ machinery, unless isogenic LcnD is present in the membrane.

3.4. LcnD, LcnD* and LcnC are organised in a membrane-associated complex

Formaldehyde cross-linking analysis of membrane fractions of MG1363(pKV4) cells expressing the LcnC/D secretion machinery from the plasmid was performed to examine whether LcnC and LcnD interact.

On a Western blot probed with anti-LcnD polyclonal antibodies three additional bands were observed in the membrane fraction of formaldehyde-treated MG1363-(pKV4) cells (Fig. 4A). None of these bands were, as expected, present in the extracts of MG1363. Molecular size comparisons suggest that the three bands may be due to interactions between LcnD and LcnD* (95 kDa), between two molecules of LcnD (110 kDa), and between two molecules of LcnD and one LcnC molecule (200 kDa). After anti-LcnD antibody stripping and reprobing with anti-LcnC polyclonal antibodies, a 200 kDa band cross reacted (Fig. 4B). This could be either a 2LcnD–LcnC complex, like it was in panel A, or an LcnC dimer, which would have approximately the same molecular masses. In addition, the anti-LcnC antibodies detected a band that was a few kDa larger than that of LcnC (Fig. 4B). This band may be the product of interaction between LcnC and LcnA. The latter could not be confirmed as, unfortunately, it has not been possible to make polyclonal nor monoclonal antibodies against LcnA due to the low immunogenicity of the bacteriocin (unpublished results).

In conclusion, secretion of LcnA is dependent on the two secretory proteins LcnC and LcnD. The specificity

Table 2
LcnA activity of strain IL1403 and its transformants containing the various plasmids

Strain	Activity (AU)
IL1403	n.d.
IL1403(pKV4)	5120
IL1403(pMG36A)	1280
IL1403(plcnD)	1280
IL1403(pL46)	1280
IL1403(plcnDΔ)	160

n.d., not detectable

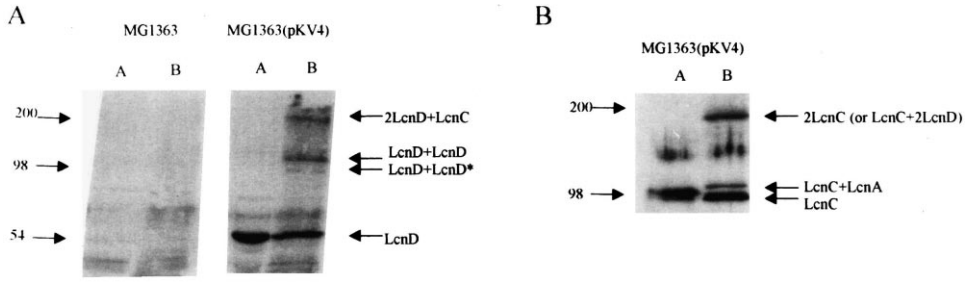

Fig. 4. Western hybridisation of formaldehyde-treated cells (late exponential phase) of *L. lactis* MG1363 (A) and MG1363(pKV4) (A,B). After the treatment, cells were fractionated and the membrane fractions (4 μg of proteins) were separated by SDS–(7.5%)PAGE. A: membrane fractions of untreated cells. B: membrane fractions of formaldehyde-treated cells. Polyclonal antibodies against LcnD (A) and LcnC (B) were used as primary antibodies. Sizes of proteins, as determined from a comparison with the protein molecular mass marker on the same gel (not shown), are indicated in the left margin.

of LcnA production is also dependent on the presence of a cytoplasmically located LcnD* protein which originates from the same mRNA as LcnD but from a different internal translational start codon. The role of LcnD* in lactococcin secretion is still unknown. It may stabilise pre-LcnA, as proposed for other chaperones in type II or III secretion systems [17], or it could keep pre-LcnA in a secretion competent state, behaving like a molecular chaperone [18]. The results of chemical cross-linking analysis provide evidence for interactions between the dedicated export proteins of the LcnA secretion system.

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